Dynamic In Vivo Imaging of Mammalian Hemato-Vascular Development Using Whole Embryo Culture

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1 Abstract

The yolk sac is the initial site of hematopoiesis in the mammalian embryo. As the embryo develops, blood vessels form around primitive erythroblasts to connect the yolk sac to the embryo, delivering newly formed blood cells to the embryonic circulation. The limited accessibility of the mammalian embryo has made it difficult to study the dynamic changes in cellular development during the formation of the early hemato-vascular system. Therefore, we have developed a culture system for studying early hematopoesis, vasculogenesis and angiogenesis in the mouse embryo. Early embryos (E7.5-E9.5) can be grown on the microscope stage in order to study the dynamics as vessels form and circulation begins. In addition, this mouse embryo culture system provides an excellent model for understanding the interplay between flow dynamics and cellular development.

2 Introduction

The mouse hematopoietic system begins to differentiate from the mesodermal germ layer just after E6.5 (1, 2). The first site of hematopoiesis, or blood formation, in mammalian embryos is the yolk sac. Clusters of primitive erythroblasts surrounded by endothelial cells emerge from extraembryonic mesoderm as "blood islands." The blood islands initially appear in an extraembryonic ring proximal to the embryo proper, and then expand to cover the entire yolk sac surface, forming the capillary plexus (3). As the heart begins to contract, around E8.5, vascular channels become perfused and the red blood cells lose begin to circulate within the vessel (4). During the first few days of blood circulation, the primary plexus is remodeled, into a mature vascular network. Thus, there

is a window of development, from E7.0 to E9.5, in which most primitive hematopoiesis and vasculogenesis in the yolk sac is established.

Because the early mammalian embryo develops *in utero* and is inaccessible to experimental manipulation, much of what is known about early blood and endothelial cell differentiation has been gleaned from cells in culture or from observations of embryos made at static time points in development. Although this approach has been successful in identifying key candidate molecules involved in cell fate decisions, this paradigm is not well-suited for studying the dynamic events in hemato-vascular development. Thus, we have developed a method to study these events in living, intact mouse embryos. Using the embryo culture technique described here, it is possible to image normal development from E7.5 until E9.5 (5), which is ideal for studying early cardiovascular development.

3 Materials

3.1 Media

1. Dulbecco's Modified Eagle Medium (D-MEM)/F-12 Media (Gibco; Carlsbad, CA, Cat. No. 11330032;).
2. Heat-inactivated fetal bovine serum (Gibco; Carlsbad, CA, Cat. No. 16140063).
3. Penicillin-Streptomycin solution (Irvine Scientific; Santa Ana, CA, Cat. No. 9366).
4. HEPES buffer solution 1M (Irvine Scientific; Santa Ana, CA, Cat. No. 9319).
5. 3 mL syringes.
6. 0.2 μm Acrodisc Syringe Filters (VWR; West Chester, PA, Cat. No 28144-040).

3.2 Embryo Dissection

1. CD-1 mouse breeding pairs.
2. CO_2 euthanasia chamber.
3. Pair of watchmaker forceps, #5.
4. 60 x 15 mm Petri dishes.
5. Heated dissecting scope: this can be achieved using a chicken incubator heater (Lyon Electric Company; Chula Vista, CA) to blow hot air over the dissection area.

3.3 Rat Serum Preparation

1. Male rats, approximately 20, at least 8 weeks old.
2. Vacutainer tubes (Becton-Dickinson; Franklin Lakes, NJ, Cat. No. 366512).
3. Venous Access Butterfly needle (Becton-Dickinson; Franklin Lakes, NJ, Cat. No. 367283).
4. Absorbent Bench Protector.
5. Rat Guillotine (Braintree Scientific; Braintree, MA, Cat. No. RG-100).
6. Ether.
7. 50 mL Falcon tubes (Becton-Dickinson; Franklin Lakes, NJ, Cat. No. 352098).
8. 0.45 μm Acrodisc Syringe Filters (VWR; West Chester, PA, Cat. No 28144-007).

3.4 Culture Chamber Components

1. Nunc Lab-tek 2-well, chambered coverglass (Nalge Nunc; Rochester, NY, Cat. No. 155380).

2. Soldering iron.
3. Mineral oil, embryo-tested (Sigma; St. Louis, MO, Cat. No. M8410).
4. 3/16"" Silicone Airline Tubing (Hagen; Holm, Germany, Cat. No. 11127)
5. Barbed Polypropylene fitting, reducing connector, 1/16" to 1/8" (Cole-Parmer Instrument Co.; Chicago, IL, Cat. No. EW06365-44).
6. Teflon tape.
7. Silicon grease (Dow Corning; Midland, MI).
8. Gas-washing bottle (Fisher Scientific; Springfield, NJ).
9. Pressurized Cylinder with regulator with a 5% CO_2, balance air mixture of gas.

3.5 Microscope and Heater Box Components

1. Inverted microscope equipped for time-lapse imaging.
2. Cardboard (~4mm thick).
3. 5/16” thick, foil-foil insulation thermal insulation (Reflectix Co.; Markleville, IN).
4. Velcro adhesive-backed strips (McMaster-Carr; New Brunswick, NJ, Cat. No. 9273K33)
5. Egg-incubator heater (Lyon Electric Company; Chula Vista, CA, Cat. No. 115-020)

4 Methods

4.1 Rat Serum Preparation

This procedure is modified from Hogan et al. (6). Note that male rats are used, as female rat blood contains hormones that reduce viability of embryos in culture. Ether is

the recommended anesthetic because it evaporates completely from the blood once it is collected. Trace amounts of anesthetics can impair embryonic growth.

1. Anesthetize male rats by placing them in a bell jar containing ether. Once the rats are sedated, remove them from the bell jar and place them on their back on a square sheet of bench protector in a fume hood. Place a 50 mL falcon tube containing a piece of ether-soaked paper towel over the rat's nose to keep it sedated.
2. Spray the abdomen with 70% ethanol and make a v-shaped incision into the lower abdomen.
3. Move the intestines to expose the dorsal aorta, which can be identified as a small (around 1 mm diameter) pulsing artery next to the larger, darker vena cava.
4. Puncture this artery using the butterfly needle. Venous Access butterfly needle consists of a butterfly needle connected by tubing to a normal needle. Once blood is seen in the tubing, the other needle should be thrust into a vacutainer tube in order to create a mild suction. This procedure requires two people since the butterfly needle needs to be held very still to enable good flow.
5. Invert the vacutainer tubes a few times during blood collection to prevent clotting. With practice, 7-8 mL of blood per rat can be obtained.
6. After collection, place the blood sample on ice.
7. Sacrifice the rat, and place it in a waste bag. It is convenient to collect bloof from approximately twenty rats per session, yielding around 50 mL of serum.
8. Carcasses must be left in a fumehood overnight to allow the ether to evaporate, particularly if the animal facility incinerates animal waste.
9. After collection, centrifuge the blood at 1300 x g for 20 minutes at room temperature.
10. Remove and pool the supernatants in 50 mL Falcon tubes.

11. Centrifuged the serum at 1300 x g for 10 minutes to remove remaining cells.
12. Heat-inactivate the serum at 56°C for 30 minutes with the lid of the Falcon tube partially unscrewed to allow the ether to evaporate.
13. Slowly filter the serum using a 0.45 μm filter and aliquot into 1 mL samples.
14. Store in a -80°C freezer for up to one year.

4.2 Media Preparation

Two types of media are prepared for the static culture technique: a dissecting medium and a culture medium. Media can be prepared the night before, if desired, and stored at 4°C.

4.2.1 Dissection Medium

The dissection medium is composed of 90% DMEM/F-12, 1% HEPES solution, 8% heat-inactivated FBS, and 1% Penicillin-Streptomycin. About 50 mLs of dissection medium is needed per mouse. The FBS and pencillin-streptomycin can be frozen together in aliquots to speed up the media preparation; however the HEPES solution should be added fresh. The medium is placed in a water bath at 37°C for at least a half hour prior to use.

4.2.2 Culture Medium

The culture medium is 49%DMEM/F-12, 49% heat-inactivated rat serum (as described in section 4.1), 1% Pencillin-Streptomycin solution and 1% HEPES solution. Two mL of medium are required for a single 21x20mm well of a 2-well Labtek Chamber slide, with

up to three embryos per well. The medium must be sterile-filtered using a 3mL syringe and a 0.2 μm filter. The tube of medium is then placed in a tissue culture incubator with the lid unscrewed at 37°C for at least an hour, to allow temperature and pH to equilibrate.

4.3 Dissection

1. Set up male and female breeder mice overnight. The presence of a vaginal plug in the morning is considered E0.5.
2. On the E7.5, E8.5 or E9.5, euthanize females with CO_2. Make a v-shaped incision in the lower abdomen and remove the uterine horns. Place these in a petri dish and cover with warmed dissection media.
3. Dissect the embryos from the horn in a hood heated to 37°C using a chicken incubator heater (Figure 1). Techniques for dissection of embryos of various ages have been described elsewhere (7). A small amount of deciduum at the proximal end of the yolk sac should be left in place. Leave the yolk sac intact for E8.5 embryos but remove it for E9.5 embryos. Change dissecting medium every 15 to 20 minutes to keep embryos at temperature.
4. Using a transfer pipette, move the dissected embryos into Lab-tek chambers, 3 embryos per well. Add 2 mL of culture medium per well. Place the chamber into the tissue culture incubator for one hour. Heart rates tend to decrease slightly during dissection since medium is not kept perfectly at 37°C. The time in the tissue culture incubator allows the heart rate to return to normal.

4.4 Embryo Immobilization

Before imaging the embryos, it is necessary to partially immobilize the E8.5 embryos. At this stage, the yolk sac is more buoyant than the deciduum, causing the embryos to float. They also become very sensitive to small currents in the media. This is not a concern for earlier stages, in which the yolk sac has not yet expanded. Later stage embryos (E9.5) are not cultured with the yolk sac and are relatively static.

The embryos are quite sensitive to the method by which they are immobilized. Any attempt to "encase" the embryos, for example using nitex grating or agar, will exert pressure on the yolk sac, stopping circulation and impeding hemato-vascular development in the yolk sac. Two methods can be used for immobilization. First, a human hair can be tied in a knot around the deciduum and then used to prop up the embryo in the correct position (figure 2). Alternatively, an oil micrometer attached to a holding pipette can be used to hold and orient the embryo. The holding pipette requires more effort to set up, but allows a greater amount of control of the embryo.

4.5 Microscope

The choice of microscope for hemato-vascular imaging depends on the application and the mode of contrast. Stereo or compound microscopes with transmitted or reflected illumination from a halogen lamp can be used if enough inherent contrast is present in the tissue; however, this is rarely the case. Therefore, most of our imaging work has been performed using a confocal or epifluorescence microscopes in conjunction with a fluorescent label. If fluorescence microscopy is used, care must be taken not to overexpose the embryos, as the UV or visible range light used for fluorescence excitation can be harmful to the embryos if used for prolonged periods. A confocal

microscope with an automated shutter offers precise control over illumination, but microscopes using conventional epifluorescence illumination can be fitted with shutters, triggered by image collection software, to prevent overexposure when images are not being collected.

For time-lapse imaging, we use an inverted microscope. The Lab-tek chambers in which the embryos are cultured have coverslip bottoms, a feature that permits the use of either dry, low magnification objectives, as well as high magnification, high numerical aperture, oil or water immersion objectives for maximum resolution of single cells. However, the higher the magnification, the more difficult it is to keep the same field of view in focus due to the movement of the embryo. Typically, we use 5x to 20x objectives for imaging which provide a field of view of 0.2 mm^2 using the LSM 5 PASCAL confocal microscope. Smaller fields of view may require re-alignment of the image to compensate for x-y shifts and/or an autofocus protocol to maintain the plane of imaging along the z-axis.

4.6 Environmental Control

The most important factor in maintaining normal embryonic development in culture is control of the local environment. In order for mammalian embryos to develop normally, it is necessary to create a heated and humidified atmosphere, with appropriate gas transfer. Thus, maintaining temperature control, preventing evaporation and regulating gas exchange in the chamber are the most critical aspects of this culture method.

4.6.1 Temperature Control

The hemato-vascular system is one of the most temperature sensitive systems of the developing embryo. The heart rate is directly influenced by ambient temperature. Therefore, one of the most important factors in maintaining normal development is accurate environmental temperature control.

In order to keep the temperature constant, we prefer to construct a heater box that surrounds the stage, embryo chamber and microscope optics to ensure stable temperatures and prevent against thermal drift in the optics (8). The heater box is constructed from cardboard to fit snuggly around the individual microscope. One side of the box can be fashioned to include a chicken incubator heater or the hot air from a chicken incubator heater can be targeted to the heater box using flexible aluminum duct tubing on the outlet (figure 3). Holes are cut out in the front so that the eye pieces are external to the heater box.

Once the cardboard box has been fashioned, it is covered with a layer of foiled thermal insulation. Most of the microscope boxes are made with separate sides, so that the box can easily be removed from the microscope. The insulation is attached such that it extends past the cardboard, which allows the edges to be securely sealed. Velcro can be used to connect the various sides of the box, allowing the heater box to be removed from the microscope when not in use.

The chicken incubators have a built-in temperature controller. In most experiments, we required more accurate temperature control than can be obtained with the chicken

incubators. This can be achieved using a digital temperature controller (Fischer Scientific; Springfield, NJ, Model# 11-463-47A) to maintain the optimum temperature.

4.6.2 Gas Exchange

Mammalian embryos require both oxygen and carbon dioxide during culture. We have been using a gas mixture consisting of 5% CO_2, 20% oxygen, and 75% nitrogen for stages between E7.5-E9.5. Traditionally, mouse embryos have been cultured in lower levels of oxygen (5%) until they reach E9.5 (9). In our experiments, cultures were successful at all ages using the higher oxygen concentration.

In order to deliver the gas to the sealed Lab-tek chamber, it is necessary to introduce an inlet for the gas tubing. A small hole is made in the chamber lid, using a soldering iron to melt the plastic. The wells of the Lab-tek chambers are separated by a small space which is a convenient place to introduce the gas inlet. A small barbed polypropylene fitting is inserted into the soldered hole in the chamber lid, allowing the other end to be attached to tubing which supplies the gas. Vacuum grease is used to seal the space where air is introduced between the lid and the bottom of the chamber (figure 4).

4.6.3 Evaporation Control

Evaporation is the most significant problem with early embryonic culture. Even small amounts of evaporation can cause embryos to develop abnormally. The E8.5 embryos are especially sensitive to this. Therefore, several steps need to be taken to prevent evaporation.

First, the incoming air is humidified by passing it through a bubbler, or gas-washing bottle (figure 3). The gas flow rate should also be set as low as the regulator allows since insufficient aeration is rarely a problem.

In order to prevent humidity from escaping the chamber environment, the chambers are also sealed from the outside with Teflon tape. This allows the air/carbon dioxide mixture to escape, but not the water vapors. A length of about 20-25 cm of tape is used and wrapped several times around the outside of the chamber.

For E8.5 embryos, the medium should also be covered with a thin layer of "embryo-tested" mineral oil. The mineral oil does not hinder gas exchange significantly, and prevents water from evaporating. Without mineral oil, the yolk sacs of E8.5 embryos tend to shrivel, and yolk sac circulation stops.

4.7 Time-lapse Imaging

Once the heater box has reached 37 °C, which should take around 30 minutes, the culture chamber is placed on the stage of the microscope, and the inlet gas is attached to the barbed fitting (figure 3). At this point, time-lapse imaging can begin.

Since most hemato-vascular development in mouse embryo occurs over several hours, it is sufficient to image once every 5-10 minutes. For confocal imaging, Z-sections or single frames can be taken. For blood flow dynamics, short continuous movies can be taken at each time point.

Because immobilization of the embryo is not complete, the time-lapse needs to be followed to ensure that the embryo does not shift significantly in the x, y or z planes. If this is occurring, the embryo can be re-positioned or re-focused.

Culture can be sustained for up to 24 hours for embryos at E8.5 and for 12-18 hours for E9.5 embryos.

4.8 Tissue staining

Though blood in older embryos is auto-fluorescent, no signal is seen at earlier stages, E7.5 to E9.5. A transgenic mouse line in which GFP is expressed in primitive erythroblasts (figure 5) provides specific labeling of red blood cells (10). Transgenic mice expressing fluorescent proteins are ideal for dynamic imaging because fluorescent proteins provide a bright, stable, uniform marker that can be directed to specific cells using particular promoter/enhancer combinations (see (11) for review). Using stable fluorescent lines is preferred for imaging over other techniques such as the injection of fluorescent dyes, because over-manipulation of the embryos can limit viability. At present, however, only a few fluorescent protein-expressing transgenic lines are available for imaging cardiovascular development at this stage. A Tie2-GFP mouse has been developed that shows expression in all endothelial cells from E8.5 onward (12). Expression of GFP in the Tie1-EGFP mouse is restricted to certain endothelial cells (13). The promoters for several genes involved in hematopoietic development have also been used for the production of fluorescent transgenics, including the GATA-2 promoter (14), and the Flk-1 promoter (15). The ability to visualize the development of both the endothelium and the early hematopoietic cells may help clarify the related lineage of

these two cell types and whether they indeed arise from a common precursor, the hemangioblast.

Efforts to find chemical dyes that are specific to endothelial or hematopoietic cells have been unsuccessful. Several lipophilic dyes were tried, including BODIPY Ceramide TR, DiD, DiO and Hoechst (Molecular Probes; Eugene, OR) but these did not penetrate the tissue significantly. DiD-labelled LDL has some endothelial cell specificity (16), however our attempts with the dye were unsuccessful. CellTracker Orange CMTMR (Molecular Probes; Eugene, OR) and other self-loading dyes can penetrate the tissue but are not specific, but can be used as a counterstain to provide context for specific stains.

It is possible to label cells using dye injection for cell tracking. This has been achieved for E8.5 embryos (unpublished data), and can be extended to other ages in principle. A fluorescent lipophilic dye, such as DiI or DiO (Molecular Probes; Eugene, OR), is loaded within a pulled glass needle. The dye can be injected into specific cells or specific tissues using a picospritzer, which uses pulsed air, or using iontophoresis, which uses electrical current (17).

4.9 Quantitative Analysis

Embryonic culture can also be used to quantitate various parameters during hemato-vascular development. 4D-tracking software, such as Volocity (Improvision, Lexington, MA), can perform cell counts or track cell motion in order to measure migration velocity. In our lab, we have been using the ε-globin:GFP mice (10), in combination with the line

scanning function of the confocal microscope in order to assess both blood flow rates and hematocrit in the early vasculature.

Blood flow in the early vasculature is much too rapid to be followed using whole field imaging. Blood flow in the dorsal aorta at E8.5 is around 10 mm/sec (Jones, *et al.*, unpublished data). The blood flow in the yolk sac is much slower, in the range of 1-7 mm/sec depending on stage and the diameter of the vessel (Jones, *et al.*, unpublished data). The yolk sac is the location of extensive remodeling between E8.5 and E9.5, and therefore a very interesting model for angiogenic processes. In order to image blood flow in the early yolk sac using traditional cell tracking algorithms, imaging rates of at least 400 Hz are required. To overcome the high-speed requirements, line scanning rather than whole field imaging is used. The laser is positioned perpendicular to the blood vessel diameter. In the resultant images, the red blood cells appear as streaks. The time the erythroblast took to pass over the laser line can be calculated from the number of line scans in each "streak" (Jones, *et al.*, unpublished data) and can be used to calculate the velocity of the blood.

The hematocrit, or volume percent red blood cells, affects many important features of hemodynamics, or blood fluid dynamics, and can vary dramatically in diseases such as as anemia and polycythemia. The hematocrit also affects the viscosity of the blood, and is therefore central to understanding flow dynamics in the cardiovascular system. The hematocrit of early embryos has not previously been measured because of technical difficulties in blood collection. Hematocrit is dependent on vessel size, an effect known as the Fahreus effect (18). Hematocrit can be calculated from the line scans based on the percentage of pixels within a vessel that are fluorescent. The primitive erythroblasts are labeled with GFP (10) and the vessel walls can be located with dyes that mark all

cells, such as CellTracker Orange CMTMR. The hematocrit is then calculated from the line scans by calculating the number of fluorescent pixels compared with the total number of pixels in the blood vessel. Blood flow in early vessels is parabolic (Jones, *et al.*, unpublished data) and radially symmetric, so a line scan through the center of the vessel is a good representation of the red blood cell density within the vessel. Hematocrit was found to rise from around 15% to around 30% between E8.5 and E10.5 (Jones, *et al.*, unpublished data).

By combining the culture of mammalian embryos with quantitative analysis using line scanning, we have been able to investigate aspects of the development that were previously inaccessible.

5 Notes

1. If the DMEM/F-12 has a slight pinkish color, it can still be used for dissection but not fir culture. DMEM contains a pH indicator called Phenol Red, which is pink when basic and yellow when acidic. The addition of HEPES to already basic medium means that the HEPES is being used to buffer the medium and will not be useful in buffering the changes in pH during culture. As a test, observe whether the DMEM/F-12 changes color upon addition of HEPES solution. If it does, the DMEM/F-12 should be replaced.
2. The second most common cause of failed cultures is the HEPES solution. If cultures begin to fail, try using new HEPES solution.
3. Previous protocols for rat serum production used syringes to gently pull the blood, rather than the vacutainer system. We have found that this method tends to cause red cells lysis, spoiling the serum.

4. If a heated dissection scope is not available, embryos can be kept warm by more frequent changing of the dissection medium. This approach is, however, not optimal.
5. The soldering iron used to introduce the gas will become covered with plastic and will then be unusable for soldering. If this is unacceptable, any method to introduce a small hole in the plastic lid is suitable, such as a heated syringe needle.
6. It is also possible to use vacuum grease to seal the Lab-tek chambers rather than wrapping the chamber in Teflon tape. This allows embryos to be oriented while on the microscope stage, with no lid on the chamber. When the lid is put on the chamber, vacuum grease in the inner edge of the Lab-tek lid is used to seal chamber. This process is less desirable then sealing with Teflon tape since escaping gas will carry some water vapor with it.
7. Embryos are very sensitive to photo-damage by UV light, especially at higher magnifications. If embryos do not develop normally, static culture on the microscope should first be attempted without imaging or with only white light. In this way, it is possible to ascertain whether the UV light is harming the embryos. If so, reduce the magnification, the number of images acquired, the laser power (for confocal), or ideally, switch to a multi-photon microscope.
8. If imaging is performed from above the culture, it must be kept in mind that even slight pressure on the yolk sac causes circulation to terminate, altering hematopoietic development. It is possible to use dipping lenses if upright imaging is necessary. However, care must be taken to ensure a sealed environment, and constant refocusing will be necessary as the yolk sac expands. The expansion of the yolk sac is less of an issue on inverted microscopes.

9. Embryos that develop in static culture should always be scored for hallmarks of normal development after imaging. This is necessary because not all embryos will develop successfully in static culture (5).
10. Imaging may be hindered by the yolk sac at E8.5 or by the turning motion of the embryo due to axial rotation. We have had some success culturing embryos without their yolk sac.
11. Embryos as early as E5.5 can also be cultured using this system. Scoring of developmental hallmarks in these embryos is especially critical.

6 References

1. Baron, M., *Induction of embryonic hematopoietic and endothelial stem/progenitor cells by hedgehog-mediated signals.* Differentiation, 2001. **68**(4-5): p. 175-85.
2. Palis, J. and M.C. Yoder, *Yolk-sac hematopoiesis: the first blood cells of mouse and man.* Exp Hematol, 2001. **29**(8): p. 927-36.
3. Yancopoulos, G.D., M. Klagsbrun, and J. Folkman, *Vasculogenesis, angiogenesis, and growth factors: ephrins enter the fray at the border.* Cell, 1998. **93**: p. 661-664.
4. Haar, J.L. and G.A. Ackerman, *A phase and electron microscopic study of vasculogenesis and erythropoiesis in the yolk sac of the mouse.* Anat Rec, 1971. **170**(2): p. 199-223.
5. Jones, E.A., et al., *Dynamic in vivo imaging of postimplantation mammalian embryos using whole embryo culture.* Genesis, 2002. **34**(4): p. 228-35.
6. Hogan, B., et al., *Manipulating the mouse embryo : a laboratory manual*. 2nd ed. 1994, Plainview, N.Y.: Cold Spring Harbor Laboratory Press. 497.

7. Nagy, A., *Manipulating the mouse embryo : a laboratory manual*. 3rd ed. 2003, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. cm.

8. Kulesa, P.S. and S.E. Fraser, *Neural crest cell dynamics revealed by time-lapse video microscopy of whole embryo chick explant cultures.* Developmental Biology, 1998. **204**: p. 327-344.

9. Copp, A.J. and D.L. Cockroft, *Postimplantation mammalian embryos : a practical approach*. The Practical approach series. 1990, Oxford ; New York: IRL Press. 357.

10. Dyer, M.A., et al., *Indian hedgehog activates hematopoiesis and vasculogenesis and can respecify prospective neurectodermal cell fate in the mouse embryo.* Development, 2001. **128**(10): p. 1717-30.

11. Hadjantonakis, A.K., et al., *Technicolour Transgenics: Imaging Tools for Functional Genomics in the Mouse.* Nature Reviews Genetics, 2003. **4**: p. 613-625.

12. Motoike, T., Loughna, S., Perens, E., Roman, B.L., Liao, W., Chau, T.C., Richardson, C.D., Kawate, T., Kuno, J., Weinstein, B.M., Stainier, D.Y. & Sato, T.N., *Universal GFP reporter for the study of vascular development.* Genesis, 2000. **28**: p. 75-81.

13. Iljin, K., et al., *A fluorescent Tie1 reporter allows monitoring of vascular development and endothelial cell isolation from transgenic mouse embryos.* FASEB Journal, 2002. **16**(13): p. 1764-74.

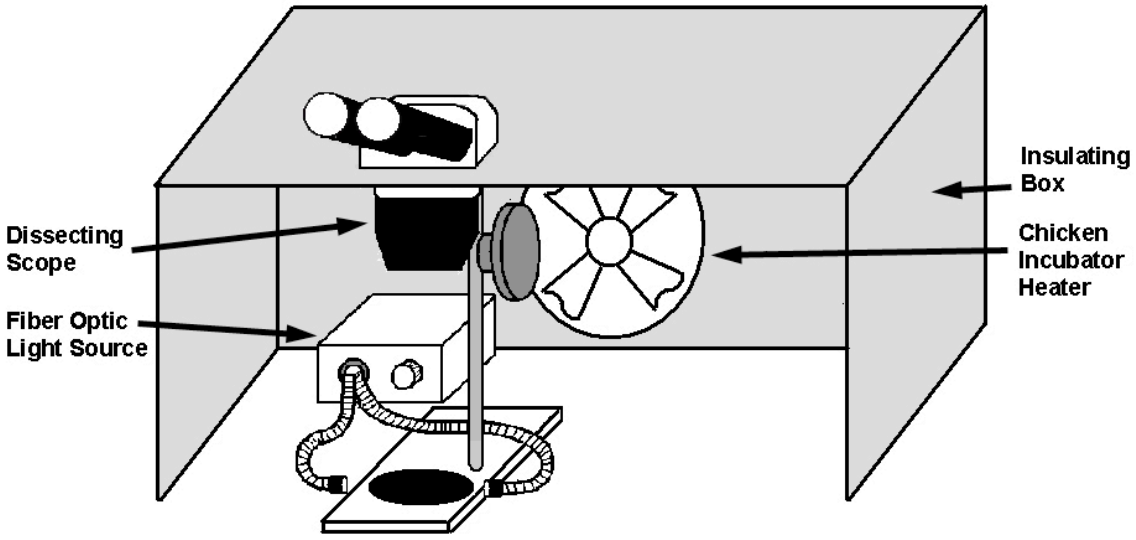
14. Minegishi, N., et al., *The mouse GATA-2 gene is expressed in the para-aortic splanchnopleura and aorta-gonads and mesonephros region.* Blood, 1999. **93**(12): p. 4196-4207.

15. Hirai, H., et al., *Hemogenic and nonhemogenic endothelium can be distinguished by the activity of fetal liver kinase (Flk)-1 promoter/enhancer during mouse embryogenesis.* Blood, 2003. **101**(3): p. 886-93.

16. Bkaily, G., et al., *The use of confocal microscopy in the investigation of cell structure and function in the heart, vascular endothelium and smooth muscle cells.* Molecular & Cellular Biochemistry, 1997. **172**(1-2): p. 171-94.

17. Quinlan, G.A., P.A. Trainor, and P.P. Tam, *Cell grafting and labeling in postimplantation mouse embryos.* Methods in Molecular Biology, 1999. **97**: p. 41-59.

18. Fahraeus, R. and T. Lindqvist, *The viscosity of blood in narrow capillary tubes.* Am J Physiol, 1931. **99**: p. 563-568.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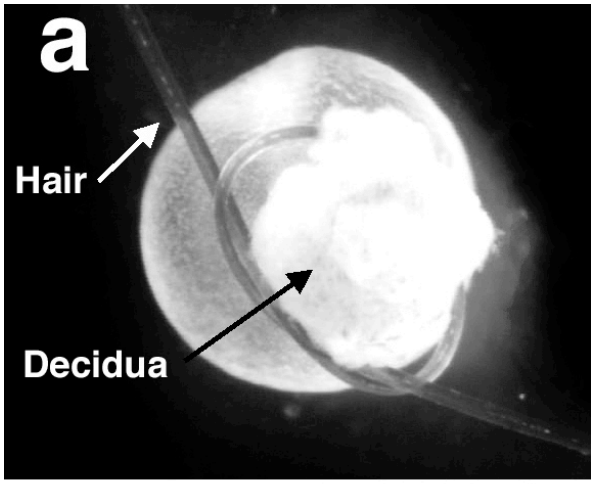
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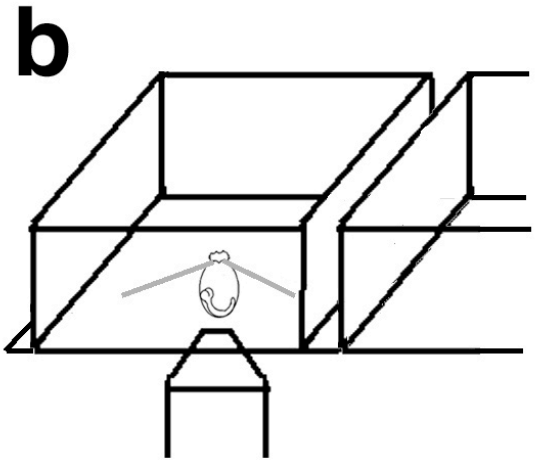
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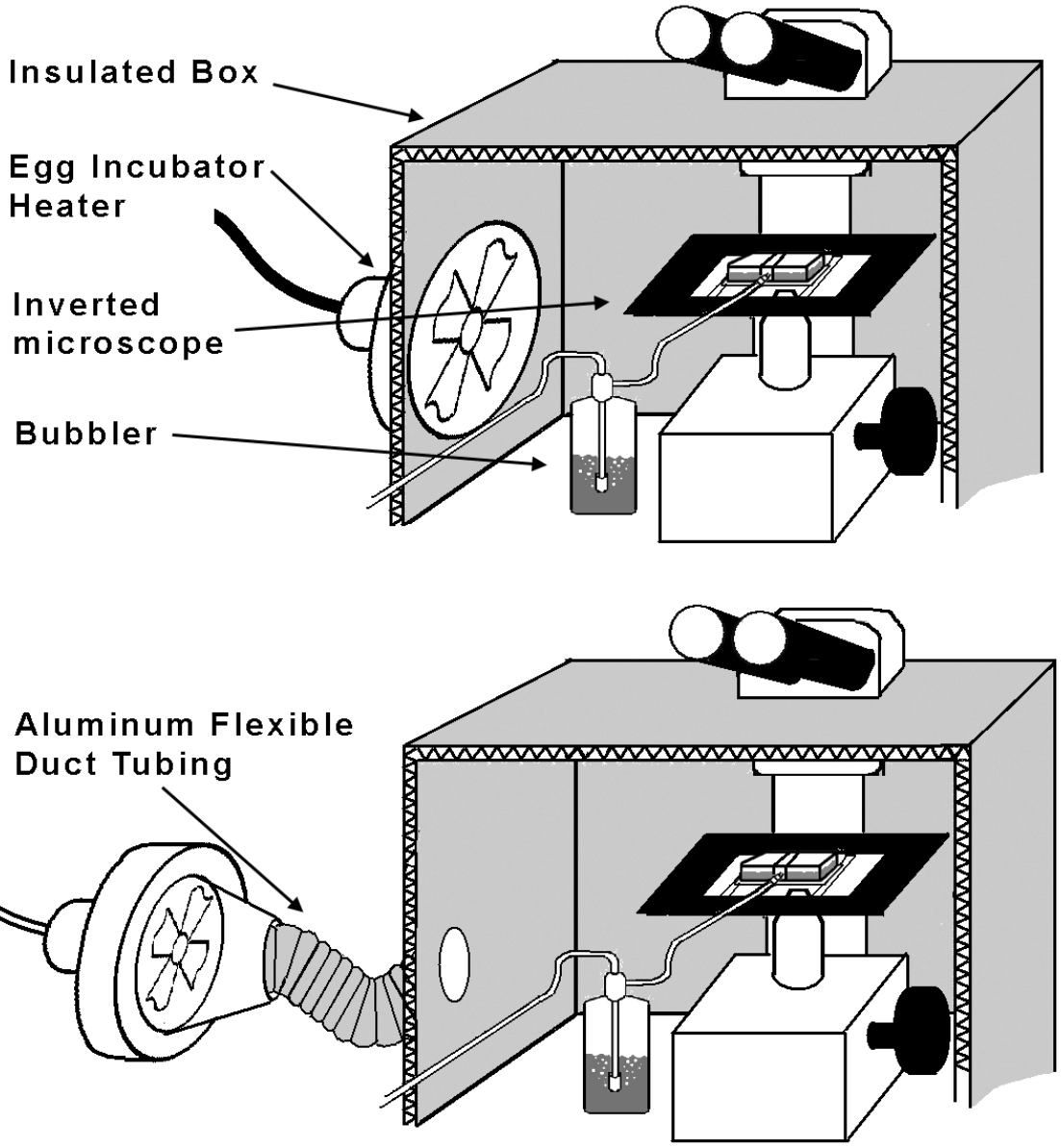
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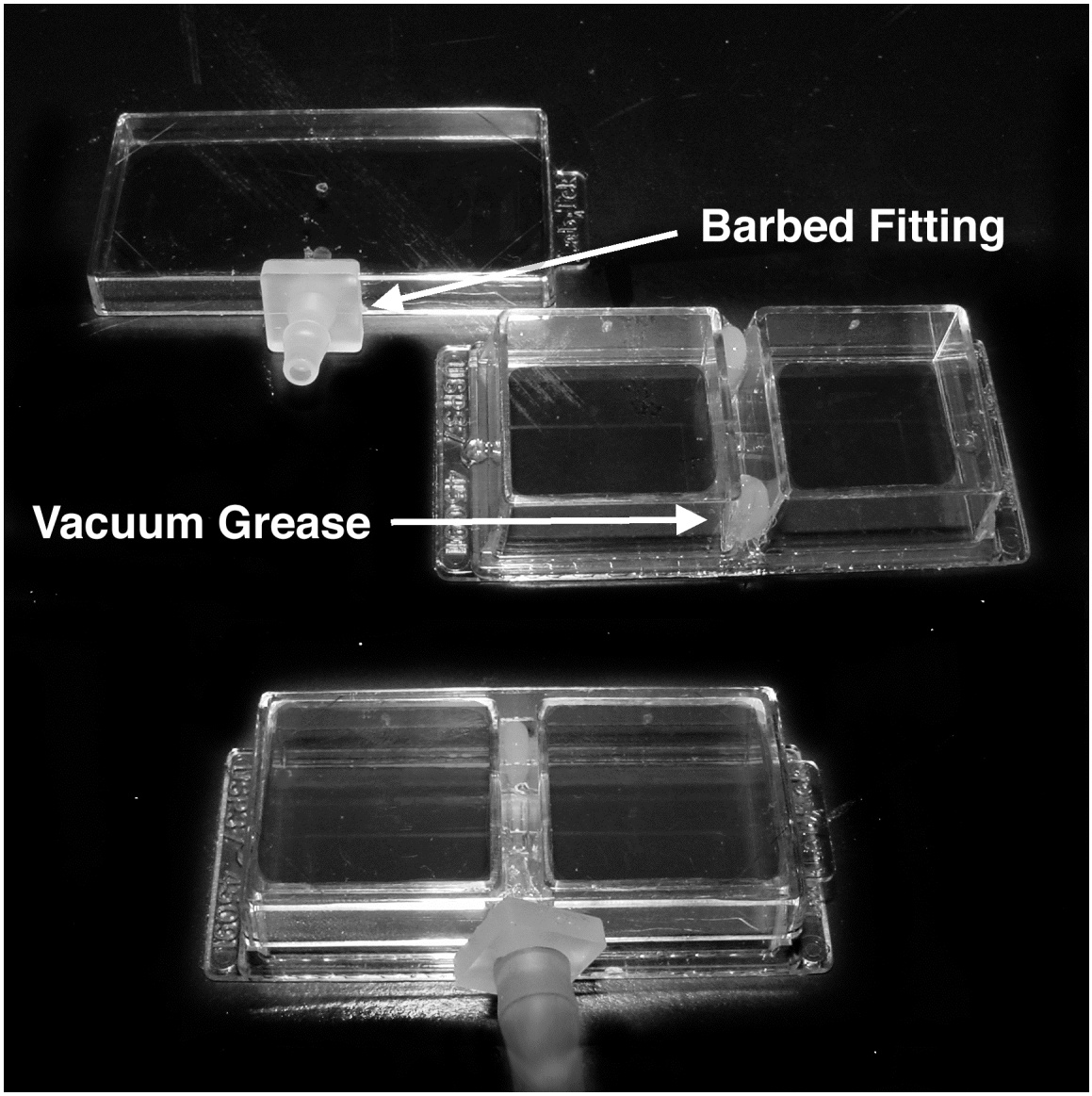
Bubbler
Aluminum Flexible Duct Tubing


Barbed Fitting
Vacuum Grease

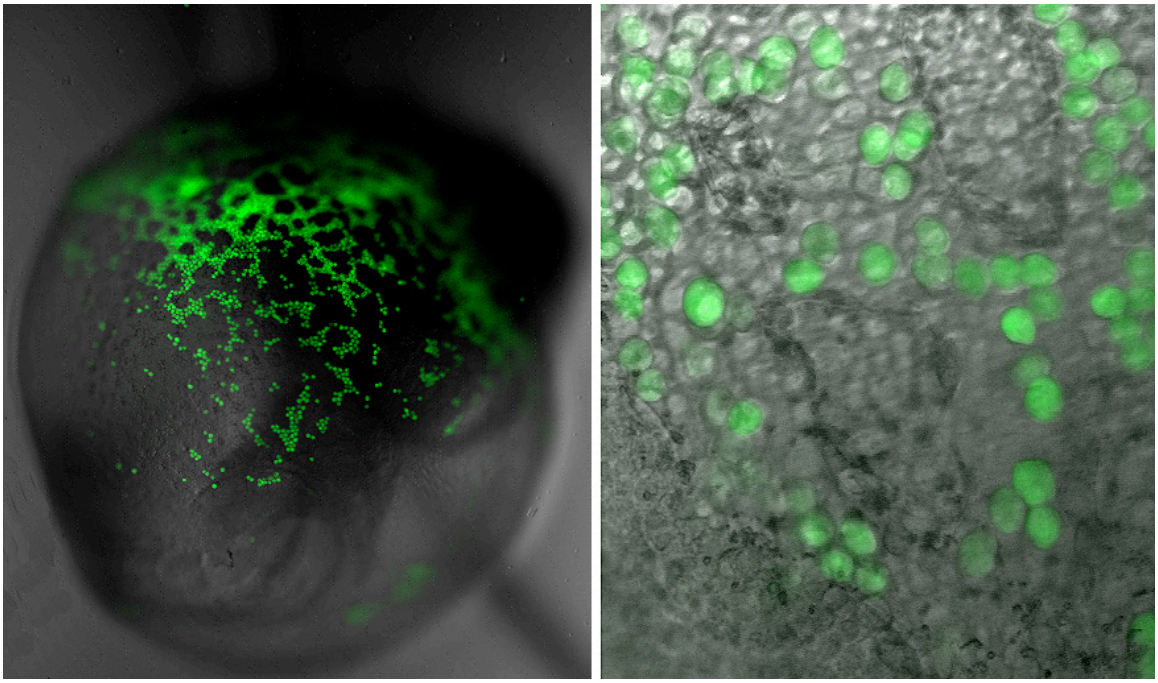


Figure Legend

Figure 1 – Heated Dissection Hood. Dissection microscope can be maintained at 37°C using a chicken incubator heater and an insulated box. The front of the heater box does not need to be enclosed.

Figure 2 – Immobilization of E8.5 Embryos. E8.5 embryos are immobilized by leaving a small amount of decidua on the ectoplacental cone and tying a piece of human hair around that decidua. The embryos are then positioned on the microscope stage such that the tissue of interest is in view.

Figure 3 – Microscope Configuration. The heater box is constructed around the microscope (shown with front panel cut-away). The box is kept at temperature with a chicken incubator heater. This heater can either be incorporated as one of the heater box walls (top) or placed in a separate location and connected to the box using aluminum ducting (bottom). The inlet of the air needs to be humidified through a gas-washing bottle and connected to the Lab-tek chamber using flexible tubing and a barbed polypropylene fitting.

Figure 4 – Modification and Aeration of Lab-tek Chambers. A small hole is made in the Lab-tek chamber lid and a barbed polypropylene fitting is inserted (top left). The space between the chambers is filled with silicon grease to seal chamber (top right). The lid is placed on top of the chambers and gas is fed to fitting using flexible tubing (bottom).

Figure 5 – Expression of GFP in Primitive Erythroblasts. Expression of GFP can be seen in the individual erythroblasts of the blood islands in E8.5 embryos (A) and in flowing erythroblasts in E9.0 embryos (B). Using the ε-globin promoter to drive GFP expression, fluorescence is restricted to red blood cells.